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Effects of *Colletotrichum capsici* infection on the growth and antioxidative response on defense mechanisms of *Capsicum annuum*

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ABSTRACT

Aims: *Capsicum annuum* (pepper) is one of the most important crops in the world. It contains an impressive list of phytonutrients that were found to have disease-preventing and health-promoting properties. Today, they are grown widely in many parts of the world as an important commercial crop. However, fungal infection is the main problem that leads to the common pepper disruptive disease, known as the Anthracnose, which lowers the yields of this plant. Therefore, this study was conducted to determine the growth, enzymatic antioxidant specific activities of catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (g-POD) as well as non-enzymatic antioxidants including ascorbic acid, α-tocopherol and carotenoids content in *C. annuum* as a response to the fungal infections by *Colletotrichum capsici*.
Methodology and results: The assays were conducted at 0, 1, 2, 3, 4 and 5 weeks of fungal treatment periods. The results showed that plant growth (plant height, leaves number, length and width, chlorophyll content) was significantly lower in treated plants compared to controls. The CAT specific activity increased drastically at the early stages of the experiment and decreased thereafter. In contrast, the APX and g-POD specific activity were initially lower, but increased significantly at the later stages of the experiment. For the non-enzymatic antioxidants, the amount of ascorbic acid, α-tocopherol and carotenoids content were significantly higher at the first week of treatment and slowly reduced afterwards.
Conclusion, significance and impact of study: This study shows that fungal infection inhibited the growth of pepper plants and the enzymatic and non-enzymatic antioxidants worked in concert to fight against the stress caused by the fungal infection, with their different specific roles in removing and reducing the reactive oxygen species in stress condition.

Keywords: Capsicum annuum, *Colletotrichum capsici*, growth, chlorophyll content, antioxidants

INTRODUCTION

Capsicum annuum L. is one of the most important economic food crops grown worldwide for domestic usage and export. It can be used as a vegetable and also as spice (Kambar *et al.*, 2014). Pepper fruits contained a broad variety of phytochemicals including carotenoids, flavonoids, phenol, ascorbic acids, capsaicin, and other components that can determine the high variability in smell, flavour, size and taste in fruits, and consequently influences consumer preference (Navarro *et al.*, 2006; Conforti *et al.*, 2007). *Capsicum annuum* may be best known for its biosynthesis of capsaicinoids, a family up to 25 related alkaloid analogs produced in epidermal cells of the fruit that account for the pungent or 'hot' sensation when consumed. Research has indicated several potential beneficial health effects of peppers, especially concerning lipid metabolism, diabetic control, digestive function and antioxidative potential (Materska and Perucka, 2005). Capsaicin, active principal in peppers, is related to enhancing energy expenditure and fat oxidation, reduced obesity and related disorders in human (Ahuja *et al.*, 2006).

In spite of its increasing demand, peppers suffer from various diseases and anthracnose is one of the most important diseases of peppers worldwide. The disease drastically reduces the yield, deteriorates the fruit quality, and hence results in low returns for farmers (Kambar *et al.*, 2014). In severe cases, the crop loss may range from 12.5 to 45% of 31, 810 tonnes total production in India (Sharma *et al.*, 2011), up to 80% of 420, 000 tonnes of its total production in Thailand (Poonpolgul and Kumphai, 2007; Montri *et al.*, 2009; Kambar *et al.*, 2014). At least 3 species of *Colletotrichum*, *Colletrotricum capsici*, *C. gloeosporioides* and *C. acutatum* have been reported as pathogens causing chili anthracnose. Out of these, *C. capsici* is the major pathogen causing this disease (Susheela, 2012; Chaisemsaeng *et al.*, 2013).

Interaction between the pathogen and plant is known to stimulate early responses in plants by producing

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reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), superoxide radicals (O_{2^-}) and hydroxyl radical ($OH^·$) which causes lipid peroxidation and subsequently membrane damage in the host plants. Thus, multiple defense lines that include both scavenging enzymes and non-enzymatic antioxidants have evolved to promptly inactivate the radicals as soon as they are formed (Sharma *et al.*, 2012). To date, reports on the effect of *C. capsici* infection on growth and defense mechanisms in chili has been scarce. Since the coordination of these multiple mechanisms is not well understood, this presents a great challenge to unravel the defense mechanisms in this species. Thus, the aims of this study were to determine the growth parameters, chlorophyll content and the defense strategies of chili plants by measuring the enzymatic (catalase, CAT; guaiacol peroxidase, g-POD and ascorbate peroxidase, APX) and non-enzymatic antioxidants (ascorbic acid, tocopherols and carotenoids content) in response to *C. capsici* infection.

MATERIALS AND METHODS

Plant materials

The seeds of *C. annuum* were obtained from Pertubuhan Peladang Kawasan Maras/Batu Rakit, KM 25 Jalan Maras, 21020 Kuala Terengganu, Terengganu. The seeds were germinated in germination tray containing 16.0 g peat-free soil medium at 0.5 cm depth. After 4 weeks, the seedlings were transferred into the polybag filled with 400 g of sandy loam soil. The plants were watered twice daily with 100 mL of water.

Fungal inoculum preparation

Colletotricum capsici pure culture was provided by Dr. Ng Lee Chuen, School of Food Science and Technology, Universiti Malaysia Terengganu. For crude fungal culture filtrates preparation, a disc of 1 cm from 8-day-old culture on Potato Dextrose Agar (PDA) was inoculated into 250 mL conical flask containing 100 mL Potato Dextrose liquid medium and incubated at 27±2 °C for 16 days to ensure an efficient growth. After incubation, the *C. capsici* culture filtrate was filtered into pre-sterilized conical flask using Whatman no. 1 filter paper. The filtrates were stored in a refrigerator at 4±2 °C for further used (Jalander and Gachande, 2012).

Fungal treatment

The 2-month-old chili plants were inoculated by drenching the soil surface in the polybags with 100 mL of the fungal culture filtrates. The experiments were conducted for 5 weeks with five replicates for each treated and control plants. The growth, chlorophyll content, enzymatic and non-enzymatic antioxidants were assayed at 0, 1, 2, 3, 4 and 5 weeks of treatment periods.

Growth parameters and chlorophyll content

The plant height was measured from soil surface to the tip of the plant using measuring tape. The leaf length and width was measured using 30 cm ruler. The chlorophyll content was determined according to the method of Harborne (1991). A total of 0.15 g fresh leaf tissues were ground up with 6 mL of 80% acetone in mortar and pestle. The homogenate was then centrifuged at 10,621 ×*g* for 10 min. The supernatant obtained was read using spectrophotometer (Shimadzu UV-1601, Japan) at 645 and 663 nm and 80% acetone was used as control.

Enzymatic antioxidant assays

(i) The g-POD specific activity was assayed according to Agrawal and Patwardhan (1993). A total of 0.15 g of leaf tissue was ground up with 1 mL of 100 mM phosphate buffer (pH 7.0) in pre-chilled mortar and pestle at 0 to 4 °C and the homogenate was centrifuged at 10,621 ×*g* (Eppendorf 5840R, Germany) at 4 °C for 10 min. The reaction mixture consists of 3 mL solution containing 1 mL 50 mM phosphate buffer (pH 7.5), 1 mL 20 mM guaiacol, 1 mL 30 mM H_2O_2 and 100 µL enzyme extract. The changes in absorbance were monitored at 470 nm for 3 min using spectrophotometer (Shimadzu UV-1601, Japan). (ii) Specific activity of CAT was assayed following the method of Clairborne (1985). A total of 0.15 g leaf tissue was ground up with 1.0 mL of 50 mM phosphate buffer (pH 7.4) and clean sand in pre-chilled mortar and pestle at 0-4°C and the homogenate was centrifuged at 10,621 ×*g* (Eppendorf 5840R, Germany) at 4 °C for 10 min. The reaction mixture contained 3 mL of reaction buffer (19 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0) and 100 µL of enzyme extract was added. The rate of changes in absorbance of the reaction mixture was monitored at 240 nm for 3 min using spectrophotometer (Shimadzu UV-1601, Japan). The CAT specific activity was expressed in µmoles of H_2O_2 consumed per minute per mg protein. (iii) Specific activity of APX was assayed based on Nakano and Asada (1981) and Sairam *et al.* (2003). Leaf tissue (0.15 g) was ground up with 1.0 mL of 100 mM phosphate buffer (pH 7.0) containing 1 mM ascorbic acid in pre-chilled mortar and pestle at 0 to 4 °C and the homogenate was centrifuged at 10,621 ×*g* (Eppendorf 5840R, Germany) at 4 °C for 10 min. The reaction mixture consists of 1.5 mL 100 mM phosphate buffer (pH 7.0), 0.5 mL 3 mM ascorbic acid, 0.1 mL 3 mM EDTA, 0.4 mL enzyme extract, 0.3 mL distilled water and 0.2 mL 1.5 mM H_2O_2 to start the reaction. The changes in absorbance were monitored at 290 nm for 3 min using spectrophotometer (Shimadzu UV-1601, Japan) and the APX specific activity was expressed as µmol ascorbate oxidized per hour per mg protein.

The crude protein concentrations were determined using method described by Bradford (1976). The Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 mL 95% ethanol. A total of 100 mL concentrated phosphoric acid was then added and the mixture volume was made up to 1.0 L with distilled water.

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The solution was filtered through a filter paper and stored at room temperature in light-proof bottles. Further, 100 µL of enzyme extract was added to 3 mL of Bradford's reagent and the absorbance was measured at 595 nm after 10 min. The protein concentrations were calculated according to a standard curve prepared with various concentrations (0 to 2 mg/mL) of Bovine Serum Albumin (BSA).

Non-enzymatic antioxidant assays

(i) α-Tocopherol was extracted based on the method by Hodges *et al.* (1996). Under dim light and over ice, 0.15 g of leaf tissue was ground up with 1.5 mL acetone and clean sand in a mortar and pestle at 0 to 4 °C. The mixture was extracted with 0.5 mL hexane followed by vortexing for about 30 sec and centrifuged at 10,621 ×*g* (Eppendorf 5840R, Germany) for 10 min. After the centrifugation, the top layer was removed and the hexane extraction was repeated twice. The assay mixture was prepared as described by Kanno and Yamauchi (1977). A total of 0.5 mL of the hexane-extract was added into 0.4 mL 0.1% (w/v) PDT (3-(2-pyridyl)-5,6-diphenyl-1,2,4 triazine) and 0.4 mL 0.1% (w/v) ferric chloride. The volume was made up to 3.0 mL with absolute ethanol and the mixture was gently swirled and left for 4 min for colour development. Following this, 0.2 mL of 0.2 M orthophosphoric acid was added to the mixture and allowed to stand for 30 min at room temperature before absorbance of the mixture was measured at 554 nm. The blank was prepared in the same manner except that absolute ethanol was used instead of the hexane-extracts. A standard curve was prepared using α-tocopherol (Sigma, type V) at various concentrations (0 to 1.4 µg/mL). The amounts of α-tocopherol in the samples were calculated based on the standard curve. (ii) Ascorbic acid was extracted according to the procedure of Jagota and Dani (1982). A total of 0.15 g of leaf sample was ground with pre-chilled mortar and pestle in 1.0 mL of 10% trichloroacetic acid (TCA) and clean sand under dim light and in ice-cold conditions. The ground sample was then centrifuged (Eppendorf 5840R, Germany) at 10,621 ×*g* for 10 min at 4°C. The supernatant obtained (300 µL) was added into 1700 µL distilled water and 200 µL of 10% Folin reagent. The mixture was gently swirled and left on bench under dim light for 10 min. Absorbance of the mixture was then measured at 760 nm. A standard curve was prepared using ascorbic acid at various concentrations (0 to 60 µg/mL). The amount of ascorbic acid in the sample was calculated based on the standard curve. (iii) Carotenoid content was analyzed according to the method proposed by Lichtenthaler (1987). Fresh leaf tissue (0.15 g) was ground up with 3 mL of 80% (v/v) acetone and clean sand in a mortar and pestle. The homogenate was centrifuged at 10,621 ×*g* for 10 min. Supernatant of the samples were measured spectrophotometrically at 663.2, 646.8 and 470 nm, while 80% acetone was used as a blank.

Statistical analysis

Statistical analysis was performed using statistical package SPSS window version 16.0. The comparisons between the growth, chlorophyll content and amount of antioxidants in treated and control plants at 0, 1, 2, 3, 4 and 5 weeks of experiments were analysed using two ways ANOVA at $P<0.05$. Data was expressed as means ± standard error. The differences were considered significant at $P\leq 0.05$.

RESULTS AND DISCUSSION

Effect of *C. capsici* on the growth of *C. annum*

Figure 1 (a to d) shows the effect of *C. capsici* infection on the growth of *C. annuum*. Similar growth trend was observed in the plant height, leaf numbers, leaf length and width throughout the experiment periods. The growth was significantly lower ($P<0.05$) in the treated plants compared to its respective controls except at 2 weeks of treatment periods. These results were in line with Berger *et al.* (2007) who stated that phytopathogen infection leads to changes in primary metabolism which affect growth and development of the plant. Upon infection, the pathogen manipulates the photosynthetic assimilates from the plant. This causes an increased demand for assimilation in the plant. The diversion of nutrients leads to the destruction of the host tissues which will inevitably lead to reduced performance and in some cases may severely cause stunted plant growth (Lucas, 2009). Phytotoxin present in the culture filtrate of *C. capsici* may also cause inhibition of plant growth and resulted in the lower number of leaves (Mathur, 1995).

Effect of *C. capsici* on chlorophyll content

The chlorophyll content in treated and control plants reduced significantly ($P<0.05$) after 2 weeks of treatment periods. However, the chlorophyll content was significantly higher ($P<0.05$) in control plants compared to treated plants from 2 to 5 weeks of treatment periods (Figure 2). Huge reduction in chlorophyll content implied that the ability of synthesizing chlorophyll dropped in cells (Bornman and Vogelmann, 1991). This might be due to the activation of the reactive oxygen species (ROS) in plant upon contact with the pathogen. ROS either disrupted photosynthetic pigment or damaged the photosynthetic apparatus causing a decrease in chlorophyll content as observed in the treated plants in this study. High ROS generation in the chloroplast may lower the potential electron transport in Photosystem II (PSII). Lower potential of electron transport caused NADP supply to decrease followed by electron leakage from ferredoxin to oxygen (O_2), reducing it to superoxide radical (O_2^-). When O_2^- is produced, it generates the aggressive ROS that lead to degradation of chlorophyll content (Mittler, 2002).

Pathogen infection can also develop chlorotic and necrotic areas leading to decrease in photosynthetic

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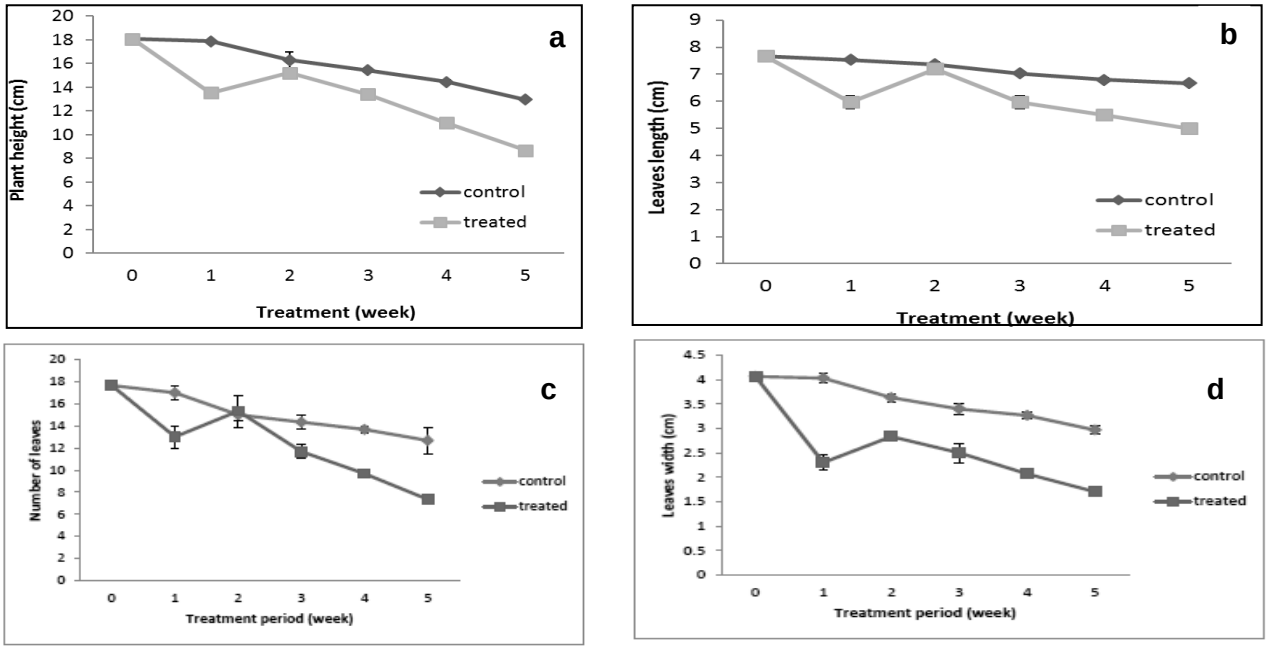

Figure 1: Changes in a, plant height (cm); ,b, leaves length (cm); c, leaves number and d, leaves width (cm) of *C. annuum* infected with *C. capsici.* Data shown are means ± standard error (n=3).

assimilate production, thus reducing the yield. Reduced chlorophyll content as observed in this study could also be due to the breakdown of chlorophyll, inhibition of chlorophyll synthesis and reduction in the number of chloroplasts (Berger *et al.*, 2007).

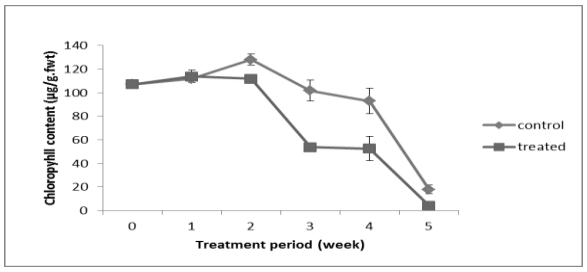

Figure 2: Changes in chlorophyll content (µg/g.fwt) of *C. annuum* infected with *C. capsici.* Data shown are means ± standard error (n=3).

Effect of *C. capsici* on enzymatic antioxidants

Figure 3 shows the changes in CAT, APX and g-POD specific activities of *C. annuum* in untreated and *C. capsisi*-treated plants. At the early stages of the experiment, the CAT specific activities in control plants were significantly higher ($P<0.05$) compared to the treated plants especially at the first week. After one week, the CAT specific activities in both control and treated plants decreased drastically (Figure 3a).

Higher CAT specific activities in control plant especially at the first week might be related to other normal processes in plant. Zentgraf (2007) reported that the most important endogenous factors inducing stress in normal plant are the ages of leaves and the developmental phases of a plant. Towards the end of the experimental period, the plant is facing the senescence process; hence, the CAT specific activity was largely decreased.

Fungal culture filtrates or other pathogen-derived selective agents or elicitors are able to induced defense responses, including oxidative stress in plants (Vidhyasekaran *et al.*, 2002). These plant-pathogen interactions triggered the excessive accumulation of ROS, mainly the hydrogen peroxide (H_2O_2) and O_2. Hydrogen peroxide plays dual role in plants: at low concentrations, it acts as a signal molecule involved in acclamatory signaling triggering tolerance to various stresses. At high concentrations, it leads to programmed cell death (PCD).

For the treated plants, longer treatment periods lead to the excessive accumulation of H_2O_2, thus, activating a variety of defense mechanisms, including the CAT activities especially at the first week of experiments. However, the CAT specific activities were decreased significantly after that as at this point, the other enzymatic antioxidants (APX and g-POD) played their roles as the ROS scavenger. In this study, the CAT specific activities was inactivated and degraded because it has a lower

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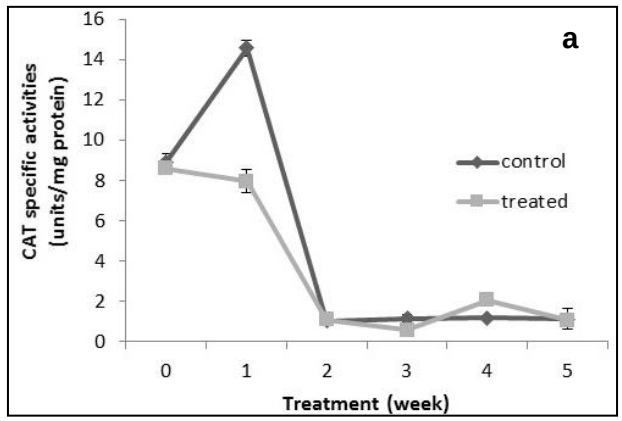

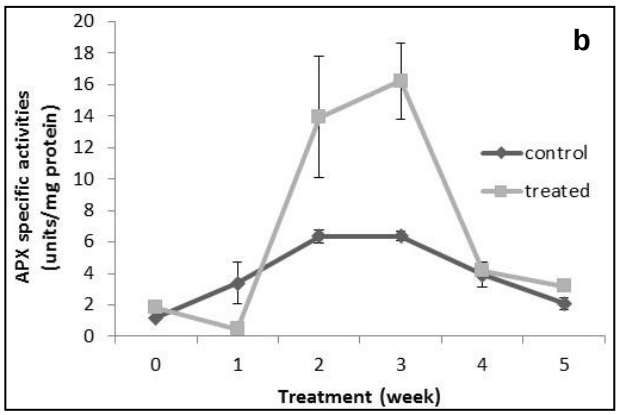

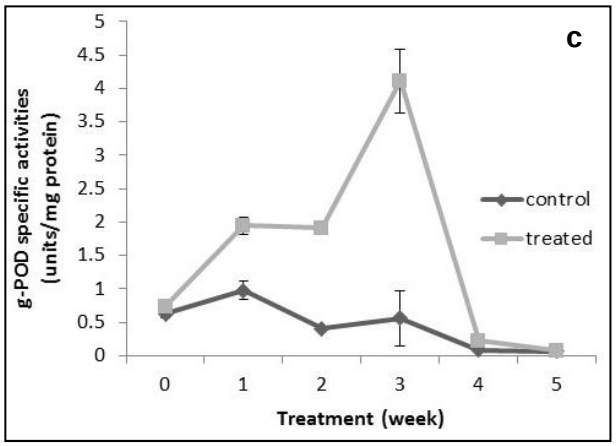

Figure 3: Changes in the a, CAT; b, APX and c, g-POD specific activities of *C. annuum* infected with *C. capsici.* Data shown are means ± standard error (n=3).

affinity for H_2O_2 compared to other enzymatic antioxidants, i.e. APX and g-POD. Gill and Tuteja (2010) reported that APX plays more crucial role than CAT and POD in managing ROS during stress because it has a higher affinity for H_2O_2. In addition, Chkhubianishvilli *et al.* (2011) observed that the decrease or inactivation of one enzyme was accompanied by the increase or activation of the other enzymes. In this study, CAT, APX and g-POD are very important enzymes that can protect cells against oxidative damage. The balance between these enzymes is very crucial to maintain ROS level under threshold values.

The changes in APX specific activities of *C. annuum* in untreated and treated plants are shown in Figure 3b. A different response was observed in APX specific activities in treated *C. annuum* plants throughout the experiment. *Colletotrichum capsici* infection significantly induced ($P<0.05$) the APX specific activities to maximum activities at 2 to 3 weeks of experiment and decreased thereafter. The APX specific activities are important in scavenging the ROS under the stress condition caused by both biotic and abiotic factors on plants. The essential role of APX in countering the increase in ROS has been extensively reviewed (Suzuki and Mittler, 2006; Ishikawa and Shigeoka, 2008; Foyer and Noctor, 2011; Gallie, 2013). Higher amount of ROS formed in treated chili plants leads to higher APX specific activities compared to control plants. According to De Gara *et al.* (2003), APX is able to reduce H_2O_2 to water by utilizing ascorbic acid as specific electron donor and it is considered as the main enzymatic systems for protecting cells against oxidative damage. In contrast, reverse trend was obtained by Gayoso *et al.* (2004) where the level of APX were found to decrease in treated plants as compared to the control when treated with *Phytophora capsisi*. The decrease in APX specific activities at the end of this study is due to the APX suppression which contributes to a reduction in the capability of cells to scavenge H_2O_2. This enables the accumulation of H_2O_2 and the acceleration of PCD of the cells. Suppression of APX activity may have an even more dramatic effect on the induction of PCD compared to CAT due to its higher affinity for H_2O_2 (Gill and Tuteja, 2010).

In addition, treatment of *C. annuum* with *C. capsici* increased the g-POD specific activities significantly ($P<0.05$). Guaiacol peroxidase (g-POD) is important in the defense mechanism against pathogens through their role in the oxidation of phenolic compounds to quinones, causing increase in antimicrobial activity. Therefore, it may be directly involved in stopping pathogen development, accelerating the cellular death of cells close to the infection site, preventing the advance of infection and/or by generating a toxic environment which will inhibit the growth of the pathogen inside the cells (Figure 3c) (Ashry and Mohamed, 2012). Similarly, POD activities was also increased during 24 h treatment followed by a stable increased level in resistant genotypes of pea cultivars against *Fusarium solani* (Svabova *et al.*, 2011).

Effect of *C. capsici* on non-enzymatic antioxidants

Figure 4 shows the changes of the amount of ascorbic acid, α-tocopherol and carotenoids content in *C. annuum* infected with *C. capsici*. The amount of ascorbic acid in both control and infected plants increased at the first week of treatment periods and sharply decreased after that. Amount of ascorbic acid was higher in control plant compared to treated plant especially at 2 weeks of

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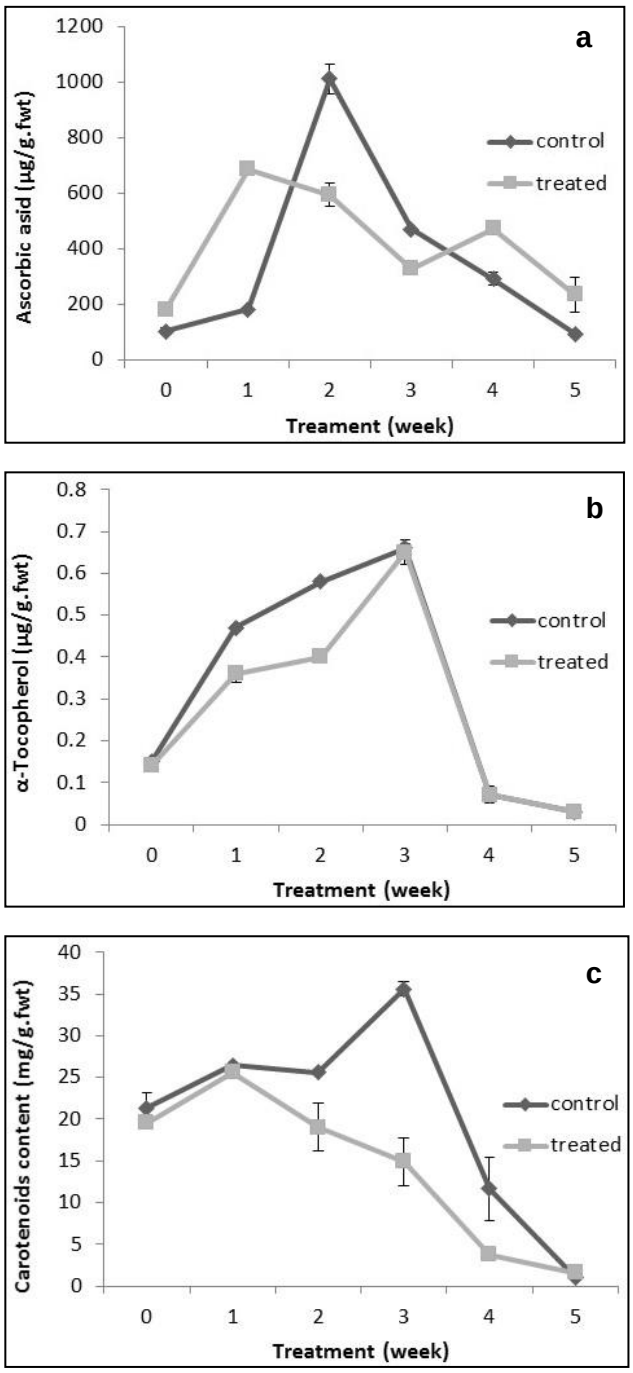

Figure 4: Changes in the amount of a, ascorbic acid; b, α-tocopherol and c, carotenoids of *C. annuum* infected with *C. capsici.* Data shown are means ± standard error (n=3).

experiment (Figure 4a). These results are in agreement with Jain *et al.* (2013) who found initial increase in the amount of ascorbic acid in pea plants infected with *Sclerotinia sclerotiorum*. Ascorbic acid is one of the major redox buffers of plant cell and is present in high amount in the chloroplast where it plays an important role in plant defence. Dikilitas *et al.* (2011) stated that ascorbic acid provides the first line of defense against ROS, and helps to protect plant cells from many factors that induce oxidative stress, including wounding, ozone, high salinity, and pathogen attack. Higher amount of ascorbic acid in infected chili plant was also observed in different genotypes of rice during bacterial blight infection caused by *Xanthomonas oryzae* pv. *oryzae* isolate DX133 (Kumar *et al.*, 2013). In contrast, Muckenschnabel *et al.* (2002) reported there was a decreased amount of ascorbic acid in *Arabidopsis thaliana* infected with *Botrytis cinerea* at early stages.

Amount of α-tocopherol increased in both treated and control plants up to 3 weeks and decreased thereafter (Figure 4b). Munne'-Bosch (2005) stated that the α-tocopherol content increased first to reduce ROS production in stressed plants where it helps in scavenging singlet oxygen formation to prevent damage in thylakoids. Besides this, α-tocopherol can prevent chain propagation in lipid autoxidation which can help in preventing further chain-carrying radicals. At the later stages, when stress is more severe, α-tocopherol content decreased significantly. This might be due to net loss of α-tocopherol since it cannot be recycled back by α-tocopherol quinone and other oxidation products (Munne'-Bosch, 2005).

Generally, the amount of carotenoids in treated plants decreased significantly after 1 week of experiment, however, the untreated plants decreased significantly after 4 weeks and afterwards (Figure 4c). In similar, Lobato *et al.* (2010) reported that *C. lindemuthianum* caused significant decrease of carotenoids in *Phaseolus vulgaris*. *Colletotrichum capsici* infection can cause oxidative stress and activates signal transduction pathways that transfer information within the cell and throughout the plant. Therefore, it may induce changes in gene expression that modify growth and development of plant and eventually affect the carotenoids biosynthesis (Othman *et al.*, 2014).

CONCLUSION

The obtained results in this study led to the conclusion that *C. capsici* infection induced oxidative stress in *C. annuum*. This pathogen-plant interaction reduced the growth and chlorophyll content, increased the enzymatic antioxidants and lowered the non-enzymatic antioxidants. These antioxidants worked together to fight against the stresses by regulating the enzymes and non-enzymatic antioxidant activities. Decrease or inactivation of CAT specific activities was accompanied by the increase or activation of the other antioxidants especially APX and g-POD specific activities as well as the low molecular weight antioxidants.

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